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NOVEL METHOD FOR THE SYNTHESIS OF NUCLEIC ACID WITHOUT PROTECTING BASE MOIETY

FIELD OF THE INVENTION

[0001]

The invention relates to a method for the synthesis of a nucleic acid without protecting a base moiety, especially to a phosphoramidite method for the synthesis of a nucleic acid oligomer using an alcohol-type compound as an activator.

BACKGROUND ART

[0002]

H-phosphonate method wherein hydroxyl group-selective condensation is carried out by means of a phosphonium-type condensing agent, BOMP, has been known for the method of synthesizing DNA without protecting a base moiety (Non-Patent Document 1). This reaction utilizes the phenomenon that an active phosphite intermediate generated during the condensation reaction will react more preferentially with a hydroxyl group than with an amino group in the base moiety.

[0003]

[Non-Patent Document 1] Wada, T.; Sato, Y.; Honda, F.; Kawahara, S.; Sekine, M., Journal of the American Chemical Society 1997, 119, 12710-12821

[Non-Patent Document 2] Gryaznov, S. M.; Letsinger, R. L., Journal of the American Chemical Society 1991, 113, 5876-5877

SUMMARY OF THE INVENTION

Problems to be solved by the invention

[0004]

However, in the above H-phosphonate method, the occurrence of such a side reaction of the base moiety as intramolecular cyclization will be exponentially increased in the DNA synthesis as the length of a chain becomes longer. As a result, it will be very difficult to prepare a desired DNA oligomer as a main component after the synthesis has proceeded to give a 12-mer. Furthermore, DNA containing a cytosine residue would cause lots of the side reaction. There has been known no effective way to prevent said side reaction.

Means for solving the problems

[0005]

The present inventors have tired to solve the above problems by forming an active phosphite intermediate in the phosphoramidite method wherein a long chain oligomer can be easily synthesized, leading to a new method for the synthesis of DNA using hydroxyl group-selective phosphorylation.

[0006]

Thus, the present invention relates to a phosphoramidite method for the synthesis of a nucleic acid oligomer with the use of an alcohol-type activator, preferably of a mixture or combination of the alcohol-type activator and an acid catalyst.

Advantages of the invention

[0007]

Up to now, the 12-mer was the longest oligomer that could be synthesized in the conventional methods without protecting the base moiety. According to the present invention, however, it is now possible to synthesize a DNA oligomer consisting of a 10-mer or a longer one, such as, for example, that consisting of a 20-mer with an extremely high purity on a solid phase. The resulting DNA oligomers may be advantageously used for a DNA chip.

Best Mode for Carrying out the Invention

[0008]

The "alcohol-type activator (compound)" in this specification means a compound that makes it possible to form the active phosphite intermediate in the phosphoramidite method, but does not mean a compound wherein a hydrogen atom of an aliphatic hydrocarbon is replaced by a hydroxyl group. Any alcohol-type activator known to those skilled in the art may be used, being preferably selected from the group consisting of hydroxybenzotriazole-1-ol (HOBt), a HOBt-derivative and a phenol analogue in order to attain a high condensation efficiency (for example, 95 % or more). The HOBt-derivative preferably has 1-4 substituents such as nitro-, bromo-, iodo-, and trifluoromethyl group, being, for example,

6-trifluoromethylbenzotriazole-1-ol, 6-nitrobenzotriazole-1-ol, or 4-nitro-6-trifluoromethyl benzotriazole-1-ol. It is more preferably that the HOBt-derivative has different substituents such as the trifluoromethyl and nitro groups at its 4 and/or 6 positions.

[0009]

Any phenol analogue known to those skilled in the art may be used, being preferably 2,4-dinitrophenol, 3,4-dicyanophenol and 2-nitro-4-trifluoromethylphenol in order to attain the high condensation efficiency as well.

[0010]

Any acid catalyst known to those skilled in the art may be used, being preferably imidazole, tetrazole and their derivatives such as, for example, benzimidazoletriflate (BIT), 4-ethylthiotetrazole, imidazolium triflate(trifluoromethane sulfonate) and 4,5-dicyanoimidazole.

[0011]

A ratio of the compounds in the combination of the alcohol-type activator and the acid catalyst may be optionally selected by those skilled in the art depending on conditions such as the kinds of each compound and reaction solvent, being usually an equivalent ratio of 1:10 - 10:1.

[0012]

The synthesis method of the present invention may be carried out in any system such as liquid or solid phase, being preferably carried out on a solid phase support for an industrial

production of the oligomers. Any solid phase support known to those skilled in the art may be used, including CPG or HCP.

[0013]

The nucleic acid according to the present invention may be DNA or RNA, which may comprise not only natural-occurring bases but also their various variants or analogues having cyclonucleoside structure in a sugar moiety or various substituents at their 2′ and/or 4′ positions. Their phosphoric acid moiety may have phosphorothionate or methylphosphonate structure.

[0014]

Various reaction conditions in the phosphoramidite method, which are not specifically described in the present specification, may be optionally selected by those skilled in the art.

Examples

[0015]

The present invention will be explained more in detail in line with the examples, which should not be construed to impose any limitations on the scope of the present invention.

[0016]

Example 1: Solid phase synthesis of dimer

Selectivity for the hydroxyl group (hydroxyl group-selectivity) in the present invention was examined by using HCP solid phase support having an end thymidine introduced thereon. Phosphorylation was carried out for one minute

between 20 equivalents of amidite units comprising each base (A, C, G, T) and 40 equivalents of various activators for said end hydroxyl group on the HCP solid phase support, followed by oxidation with 0.1 M iodine solution (pyridine: water = 9:1) for 2 min at a room temperature. A DMTr group was then removed with 3% trichloroacetic acid-CH_2Cl_2 solution for one minute at a room temperature, and a phosphoric acid-protecting group (2-cyanothyl group) was excised with ammonia for 12 hours at a room temperature.

When the conventional activator, IMT, was used, d[ApT] and d[CpT] were obtained with the hydroxyl group selectivity of 77% and 83%, respectively. On the other hand, when HOBt was used as the activator, d[ApT] and d[CpT] were obtained with the hydroxyl group selectivity of 99.7% 99.9%, respectively. The hydroxyl group-selectivity was calculated from a ratio of the area of peaks of the desired compound and N-phosphate.

[0017]

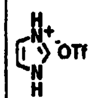
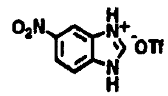
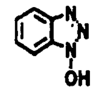
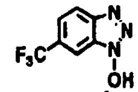
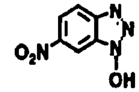
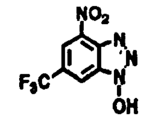
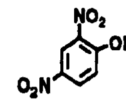
Example 2: Solid phase synthesis of trimer

Various trimers were synthesized in order to confirm utility of HOBt in the synthesis of DNA wihout protecting the base moiety. The dimmers were synthesized according to the method of Example 1 using HOBt as the activator, further followed by condensation to produce trimers. When IMT was used, the production of a considerable amount of side-products was observed in the synthesis of d[TpApT] and d[TpCpT]. The results are shown in TABLE 1. It also shows the results obtained by using NBT, an

activator for a proton-block method, in CH_3CN-NMP mixture solvent system.

[0018]

[TABLE 1]

compd	polymer	final product	% ratio of the desired product						
			IMT	NBT	HOBt	HO^{tf}Bt	HO^{n}Bt	HO^{nt}Bt	DNP
2-11a	T-HCP	ApT	77.0%	99.2%	99.7%	99.3%	98.8%	96.1%	97.1%
2-11c	T-HCP	CpT	82.9%	99.0%	99.9%	98.9%	99.8%	92.4%	99.5%
2-11g	T-HCP	GpT	>99.9%	>99.9%	>99.9%	>99.9%	>99.9%	>99.9%	>99.9%
2-4	d[ApT]-HCP	TpApT	90.5%	>99.9%	>99.9%	99.1%	97.5%	96.7%	99.6%
2-4	d[CpT]-HCP	TpCpT	9.7%	99.8%	>99.9%	98.7%	97.2%	64.7%	99.4%
2-4	d[GpT]-HCP	TpGpT	>99.9%	>99.9%	>99.9%	>99.9%	>99.9%	>99.9%	>99.9%

[0019]

Example 3: synthesis of a long chain-oligomer using a DNA synthesizer

d[CCCCCTTTTCTCTCTCTCT] and [TTAAAAATTATTAAATTATT] were synthesized by means of DNA/RNA Synthesizer 392 (Applied Biosystem Inc.(ABI)). The synthesis of the DNA oligomer was carried out using HCP solid phase support having an end thymidine introduced thereon (1 μmol, 28 μmol/g, succinyl linker) and a mixture of 0.2 M $Ho^{tf}Bt$ (6-trifluoromethylbenzotriazole-1-ol: the alcohol-type activator) and 0.2 M BIT (benzimidazoletriflate: the acid catalyst) in CH_3CN-N-methyl-2-pyrrolidone (15:1, v/v) solvent. Each elongation cycle of the synthesis is shown in TABLE 2.

[0020]

[TABLE 2]

step	operation	reagent(s)	time, (min)
1	washing	CH_3CN	0.2
2	detritylation	3% Cl_3CCOOH / CH_2Cl_2	1.5
3	washing	CH_3CN	0.4
4	coupling	0.1M amidite + 0.2M $HO^{tf}Bt$ + 0.2M BIT in CH_3CN-NMP (15:1, v/v)	1.0
5	washing	CH_3CN	0.2
6	coupling	0.1M amidite + 0.2M $HO^{tf}Bt$ + 0.2M BIT in CH_3CN-NMP (15:1, v/v)	1.0
7	washing	CH_3CN	0.2
8	oxidation	0.1M I_2 in Py-H_2O-THF (20:2:78, v/v/v)	0.5
9	washing	CH_3CN	0.4

[0021]

The DMTr group was then removed with 3 % trichloroacetic acid in CH_2Cl_2 (2 mL) for one minute, and the solid phase support was washed with CH_2Cl_2 (1 mL x 3) and CH_3CN (1 mL x 3). Finally the solid phase support was treated with conc. ammonia water (500 μL) to be excised to give a desired product.

d[CCCCCTTTTCTCTCTCTCT], Mass (M+H) calcd 5868.23, found 5869.92; Enzyme Assay dC:T = 1.00:0.99, isolated yield 79%.
[TTAAAAATTATTAAATTATT], Mass (M+Na) calcd 6130.31, found 6132.69; Enzyme Assay dA:T =1.00:0.94, isolated yield 31%.

INDUSTRIAL APPLICABILITY

[0022]

A DNA fragment having such a length as 20-mer or so is needed for an Affimetrix-type DNA chip, which is widely used for gene diagnosis. The success of the synthesis of such long DNA without protecting the base moiety has therefore made a start for a high throughput preparation of the DNA chip with a high cost performance, making a great impact on biotechnology. The present invention is the first synthesis method that can attain a practical level without protecting the base moiety. It is expected that the nucleic acid oligomers synthesized according to the present invention will be utilized in gene diagnosis such as SNP analysis.